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De novo syntheses of Marburg virus antigens from adenovirus vectors induce potent humoral and cellular immune responses

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Abstract

Marburg virus (MARV) is an African filovirus that causes a deadly hemorrhagic fever in humans, with up to 90% mortality. Currently, there are no MARV vaccines or therapies approved for human use. We hypothesized that developing a vaccine that induces a de novo synthesis of MARV antigens in vivo will lead to strong induction of both a humoral and cell-mediated immune response against MARV. Here, we develop and characterize three novel gene-based vaccine candidates which express the viral glycoprotein (GP) from either the Ci67, Ravn or Musoke strain of MARV. Immunization of mice with complex adenovirus (Ad)-based vaccine candidates (cAdVax vaccines), led to efficient production of both antibodies and cytotoxic T lymphocytes (CTL) specific to Musoke strain GP and Ci67 strain GP, respectively. Antibody responses were also shown to be cross-reactive across the MARV strains, but not cross-reactive to Ebola virus, a related filovirus. Additionally, three 1×10^8 pfu doses of vaccine vector were demonstrated to be safe in mice, as this did not lead to any detectable toxicity in liver or spleen. These promising results indicate that a cAdVax-based vaccine could be effective for induction of both humoral and cell-mediated immune responses to multiple strains of the Marburg virus.

Keywords: Marburg virus; Vaccine; Adenovirus; cAdVax; Ci67; Ravn; Musoke

1. Introduction

The Marburg virus (MARV) was the first characterized member of the filovirus family. The four species of Ebola virus (EBOV) are the only other known members of the filovirus family. Although the MARV and EBOV viruses belong to the same virus family, they are genetically distinct, and there is no antigenic cross-reactivity between them [1]. Several strains of MARV have been described, including: the Ci67 [2] and Popp strains [3] (two different isolates both derived from the first MARV outbreak of 1967 in Germany and Yugoslavia); the prototype Musoke strain from the 1980 cases in Kenya [4]; the Ravn strain from the 1987 case in Kenya [5]; and the Angola strain from the current outbreak.

The severe febrile illness caused by MARV is known as Marburg hemorrhagic fever. An RNA virus of the filovirus family, MARV is indigenous to Africa although first identified during simultaneous outbreaks in Germany and Yugoslavia. This disease can affect both humans and non-human primates; however, the animal reservoir for MARV is still unknown. Many of the signs and symptoms of Marburg hemorrhagic fever are similar to those of other infectious diseases endemic to Africa, such as malaria and typhoid fever,

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14. ABSTRACT
Marburg virus (MARV) is an African filovirus that causes a deadly hemorrhagic fever in humans, with up to 90% mortality. Currently, there are no MARV vaccines or therapies approved for human use. We hypothesized that developing a vaccine that induces a de novo synthesis of MARV antigens in vivo will lead to strong induction of both a humoral and cell-mediated immune response against MARV. Here, we develop and characterize three novel gene-based vaccine candidates which express the viral glycoprotein (GP) from either the Ci67, Ravn or Musoke strain of MARV. Immunization of mice with complex adenovirus (Ad)-based vaccine candidates (cAdVax vaccines), led to efficient production of both antibodies and cytotoxic T lymphocytes (CTL) specific to Musoke strain GP and Ci67 strain GP, respectively. Antibody responses were also shown to be cross-reactive across the MARV strains, but not cross-reactive to Ebola virus, a related filovirus. Additionally, three 1x10(8)pfu doses of vaccine vector were demonstrated to be safe in mice, as this did not lead to any detectable toxicity in liver or spleen. These promising results indicate that a cAdVax-based vaccine could be effective for induction of both humoral and cell-mediated immune responses to multiple strains of the Marburg virus.

15. SUBJECT TERMS
filovirus, Marburg, gene-based vaccine, glycoprotein, adenovirus vector, laboratory animals, mice

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and therefore makes diagnosis of the disease difficult, especially if only a single case arises. Due to the difficulty in distinguishing between these different pathogens early at the disease onset, it is important to develop an effective vaccine against this rare but fatal pathogen, especially since it has the capacity to cause swift and devastating outbreaks of disease.

MARV is composed of a helical nucleocapsid containing a negative-sense single-stranded RNA genome (19.1 kb). The viral nucleocapsid is surrounded by a membrane envelope derived from the host cell which contains a layer of surface projections (spikes) consisting of a single heavily glycosylated protein (glycoprotein; GP) [1]. The MARV precursor GP (140 kDa) is cleaved by furin or another subtilisin-like endoprotease to form functional GP1 and GP2 proteins. Mature MARV GP consists of disulfide-linked GP1 (∼160 kDa) and GP2 (∼38 kDa) subunits [6].

The MARV and EBOV filoviruses are some of the most lethal viruses known to mankind with a mortality rate of up to 90% [7,8]. While historically MARV has been less pathogenic than EBOV, its virulence appears to be evolving, as the mortality rates have risen sharply from 21% during the first MARV outbreak in 1967 to 83% and nearly 90% in the 1998 and current outbreaks, respectively. The recent MARV outbreak in Angola, which began in October 2004 and is still ongoing [7,9], has reminded us that human civilizations are still vulnerable to outbreaks of deadly diseases even in the twenty-first century. In fact, at the time of this manuscript's submission, the Angola outbreak had yet to be contained and had led to over 300 deaths from this extremely dangerous infectious disease [7,8]. It is, therefore, important that we be prepared should such an outbreak escape the borders of Africa. Additionally, the EBOV and MARV viruses have a terrifying potential as biological weapons. Dispersion of the virus in aerosols or droplets of body fluids is considered a risk, and therefore, has the potential for weaponization if a suitable stabilizing medium can be found.

Immunization against the filoviruses may play an important role in helping to prevent a natural outbreak of these diseases and to minimize their spread to the United States should such an outbreak occur in the populated regions of our neighboring countries. In addition, development of effective and rapidly deployable vaccines against these biological agents is essential to protect our troops in missions abroad and in combat situations. Vaccination of healthy individuals will also provide a source of antiserum that will be essential in rescuing infected personnel.

Traditional approaches to developing virus vaccines include immunizing individuals with either live attenuated or killed virus preparations. For safety reasons, such traditional methods for producing vaccines will not likely prove acceptable for the development of a MARV vaccine for human use. For this reason, development of a subunit vaccine against MARV has become a more preferable vaccine strategy. Ideally, such a subunit vaccine would be both effective as well as cost-efficient. To address this issue, we hypothesized that expressing MARV antigenic proteins from a complex, replication-defective adenovirus-based vaccine vector system, known as cAdVax, would induce high expression levels of neutralizing epitopes of MARV GP species, thus leading to induction of a potentially protective MARV-specific immune response. Here, we describe three candidate cAdVax-based MARV vectors, each expressing the MARV GP from individual strains of MARV: cAdVaxM(ci) (which expresses Ci67 GP), cAdVaxM(ra) (Ravn GP), and cAdVaxM(mu) (Musoke GP).

Our vaccine strategy combines a benign infection by a replication-defective adenovirus vector with the antigenic potential afforded by individual MARV strain GPs. It is our hypothesis that de novo synthesis and expression of MARV GP on cell surfaces will mimic the antigen presentation that would occur from a natural MARV infection. This would be, however, without the pathogenicity and hemorrhagic fever associated with an actual MARV infection. Theoretically, presentation of MARV antigen to the immune system should, therefore, elicit an immune response against MARV from both the humoral and cell-mediated arms of the immune system.

In this study, we found that vaccination with our cAdVax-based MARV vaccine candidates led to induction of MARV-specific humoral and cell-mediated immune responses in mice. These immune responses were cross-reactive across the different strains of MARV and were found to be specific to MARV, as these mice did not develop an immune response to the phylogenically-related Ebola virus. While other molecular genetic vaccine approaches to developing a MARV subunit vaccine are currently being evaluated [10–14], our cAdVaxM(ci), cAdVaxM(ra) and cAdVaxM(mu) vaccines are, to our knowledge, the first recombinant adenovirus-based MARV vaccines to be described.

2. Methods

2.1. Cell lines

HEK293 (human embryonic kidney) and Vero E6 (African green monkey kidney) cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA). All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone; Logan, UT). Mouse splenocytes were cultured in RPMI 1640 supplemented with 10% FBS, 10^{-5} M β-mercaptoethanol, 10 mM HEPES, 1% penicillin–streptomycin, and 0.1 mM non-essential amino acids.

2.2. Construction of the cAdVax-based MARV vaccines

The Ci67 GP (Genbank accession number AF005735; Protein ID AAC40460.1; a.a. 1–681), Ravn GP (Genbank accession number AF005734; Protein ID AAC40459.1; a.a. 1–681), and Musoke GP (Genbank accession number DQ217792; Protein ID ABA87127; a.a. 1–681) genes were

amplified by polymerase chain reaction (PCR), with each primer including specific restriction sites at the 5′ and 3′ ends for subsequent cloning of the PCR fragments into pLAd or pRAd plasmid shuttle vectors. The final PCR products were verified by sequence analyses.

In order to characterize immune responses to MARV GP proteins, we subcloned each MARV antigen into our pLAd and pRAd shuttle vectors to create a series of MARV adenovirus vaccines targeted against the Ci67, Ravn, and Musoke strains of MARV. The cAdVax-based MARV vaccine vector genomes were constructed as described previously [15–17]. All cAdVax vector genomes were based on a modified Ad5sub360 vector backbone, which contains deletions in E1, E3 and almost all E4 ORFs with the exception of ORF6.

2.3. Recombinant adenovirus vector propagation, confirmation by sequencing analysis, and titering

All vectors were propagated in HEK293 cells, using standard procedures [15–17]. Briefly, HEK293 cells, which provide Ad5 E1a and E1b functions in trans, were transfected with the recombinant cAdVax-based MARV vector genomic DNA using Lipofectamine™ Reagent (Invitrogen; Carlsbad, CA) following manufacturer's instructions. Transfected cells were maintained until adenovirus-related cytopathic effects (CPE) were observed (typically 7–14 days post-transfection), at which point the cells were harvested. After several rounds of single-plaque selection, candidate vaccine clones were confirmed by restriction map digestion as well as sequencing analysis of viral DNA isolated from positive Ad vector plaques to assure that the vaccine preparation had no deletions or rearrangements. Entire viral transgene cassettes were completely sequenced including promoter regions.

The final positive cAdVax vector clones were re-amplified in HEK293 cells and purified by ultra-centrifugation in cesium chloride gradients. Briefly, adenoviral lysates from thirty 150-mm plates were banded twice on CsCl gradients and desalted twice with PD-10 size exclusion columns (Amersham Scientific; Piscataway, NJ) into HEPES buffered saline (HBS; 21 mM HEPES, 140 mM NaCl, 5 mM KCl, 0.75 mM $Na_2HPO_4{\cdot}2H_2O$, and 0.1% (w/v) dextrose; adjust pH with NaOH to 7.5; and filter sterilize) containing 10% glycerol, and stored in liquid N_2. All vectors were titrated on HEK293 cells infected in serial dilution on triplicate columns of 12-well plates for plaque forming units (pfu). The resulting titers were scored as pfu/ml. The final vaccine was confirmed again with restriction map digestion.

2.4. Western blot

Vero cells were infected with Marburg adenovirus vaccines at a multiplicity of infection (MOI) of 100 for 48 h. Cell pellets were washed twice with phosphate-buffered saline (PBS) and lysed with lysis buffer (200 mM Tris–HCl, pH 7, with 8% Triton X-100, 2% NP-40, 20 mM NaCl and 2 mM EDTA) on ice. Cell lysates were mixed with sample buffer (50 mM Tris, pH 6.8, 2% sodium dedocyl sulfate, 1% (β-mercaptoethanol, 0.1% bromophenol blue, 10% glycerol), heated at 100 °C for 10 min, and separated on a 4–15% gradient SDS-PAGE gel (BioRad, Hercules, CA). Separated proteins were then transferred to Immobilon-P PVDF membrane (Millipore Corporation, Bedford, MA). The membrane was stained with Ponceau S for 15 min, washed with distilled water and non-specific antibody-binding sites were blocked with 5% nonfat dry milk in blocking buffer (0.05 M Tris, pH 7.5, 0.15 M NaCl, 0.01% NP-40, 0.3 mM NaN_3) for 30 min. The membrane was later incubated for 1 h with a guinea pig polyclonal Marburg virus (Ravn strain) anti-serum diluted 1:1000 in blocking buffer containing 5% nonfat dry milk. After three washes with phosphate-buffered saline containing 0.05% Tween-20, the blot was treated with horseradish peroxidase-conjugated goat anti-guinea pig immunoglobulin (Chemicon, Temecula, CA) diluted 1:10,000 for 1 h and subsequent bands were visualized using ECL Plus detection system (Amersham Biosciences, Piscataway, NJ).

2.5. Immunization of mice with cAdVax-based MARV vaccines

Four groups of 28 C57BL/6 mice (Charles River Laboratory, Charleston, SC) were immunized intra-peritoneally (i.p.) at weeks 0, 4 and 8 with 1×10^8 pfu of either cAdVaxM(ci), cAdVaxM(ra), cAdVaxM(mu), or HC4 control vector prepared in 100 μl PBS/10% glycerol. HC4, an unrelated adenovirus-based Hepatitis C vaccine, served as a negative control vaccine. At weeks 2, 4, 6, 8, 10, 12, and 24, four mice per group were sacrificed.

At sacrifice, sera were prepared to determine the antibody titer, and the splenocytes were prepared to evaluate the cellular immune responses. Each animal was analyzed independently. Vaccinated mice were visually monitored for any adverse effects resulting from immunization. Visual inspection of control and vaccinated mice was made with particular attention to food and water intake, coat texture (ruffled coats are often a sign of illness), and excessive weight loss or gain.

2.6. Serum preparation

Sera were prepared from each blood sample by incubating the blood at room temperature for approximately 4 h to allow for clotting followed by an overnight incubation at 4 °C. The following day, clots were removed and blood was centrifuged at 2000 × *g* for 10 min. Supernatants were transferred to sterile tubes and the serum stored at −80 °C. Small aliquots were stored short-term at 4 °C. Sodium azide was added as a preservative to these samples to a final concentration of 0.05%.

2.7. ELISA assay

Antibody titers of mouse sera were determined by an indirect ELISA. The antigen consisted of purified, irradiated

MARV (either Musoke, Ravn or Ci67 strain) or Ebola virus (Zaire strain) coated directly onto 96-well flat-bottom plates (Nunc-lmmuno Plate MaxiSorp Surface; Nalge Nunc International, Rochester, NY). All work involving the handling of filoviruses, including the ELISA assys, was conducted at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) in their U.S. government-approved biosafety level 4 (BSL4) facilities.

The sera from vaccinated and control mice were diluted serially in PBS/2.5% non-fat dry milk/0.5% FBS/0.025% Tween-20. Duplicate samples of each dilution were added to the prepared ELISA plates at 100 μl/well. Sera were incubated at 30 °C for 1 h. The plates were then washed three times with PBS/0.05% Tween-20. The presence of bound antibody was detected by adding 100 μl/well of 1:5000 dilution of horseradish peroxidase conjugated anti-mouse IgG H + L antibody (KPL, Gaithersburg, MD) and incubating at 30 °C for 1 h. The plates were then washed three times with PBS/0.05% Tween-20. Wells were then developed with 100 μl/well TMB-S substrate (RDI; Flanders, NJ) for 10 min. The TMB-S reaction was stopped with 100 μl/well 0.5 M HCI. OD_{450} of each well was measured using the Quant microtiter plate reader (Bio-tek Instruments Inc., Winooski, VT). Antibody titers were determined by calculating the dilution of serum that corresponded to a signal of three times the background for that particular test.

2.8. Mouse splenocyte preparations

Splenocytes were isolated from mouse spleens using 70 μm cell strainers (BD Falcon, Franklin Lakes, NJ). Red blood cells were removed using ACK lysing solution (BioSource International, Camarillo, CA). Each individual animal was analyzed independently, assaying duplicate samples of 2×10^6 splenocytes for each time point.

2.9. Antigenic peptide design and preparation

Cellular immune responses to MARV GP protein were determined by ELISpot assay using overlapping peptide pools that correspond to peptides between conserved regions of the MARV GP sequence as targets. Peptides derived from the most conserved regions of the glycoprotein (amino-acid: 28–237 and 502–681) of three different strains of Marburg virus: Ci67, Ravn and Musoke were synthetized using the Ci67 sequence.

The 15-mer consecutive peptides overlapping by 10 amino-acids were individually dissolved in DMSO at approximately 20 mg/ml. Forty peptides were synthesized from a.a. #28–237 of Ci67 GP sequence (210 a.a. = 40 peptides (15-mers)) while 34 peptides were synthesized from a.a. #502–681 of Ci67 GP sequence (180 a.a. = 34 peptides (15-mers)). Four peptide pools were generated by combining 20 overlapping consecutive peptides, except pool 4 (14 peptides), at a final concentration of 10 μg/ml and then aliquoted and stored at −20 °C. Peptide pools 1 and 2 included the homologous amino terminal region of Marburg GP1 (a.a. #28–237 of Ci67 GP), and pool 3 and 4 included the homologous carboxy-terminus of GP2 (a.a. #502–681 of Ci67 GP). A pool of similarly constructed 15-mer peptides from the E protein of dengue virus type-2 (a.a. #1–110) was used as a negative control and concanavalin-A (ConA) was used as a positive control. Peptides were used at 5 μg/ml final concentration, keeping DMSO concentration below 0.5% (v/v) in all final assay mixtures. All peptides were synthesized by Mimotopes (Victoria, Australia).

2.10. Murine IFN-γ ELISpot assay

To coat the ELISpot plates, 100 μl of 70% ethanol was added to each well of the 96-well plate (Multiscreen 96-Well Assay Plates; Millipore, Bedford, MA). After two washes with PBS, mouse IFN-γ ELISpot antibody (BD Biosciences, San Jose, CA) was added to each well and incubated at 4 °C overnight. After three washes with PBS, the plate was blocked with 200 μl/well of RPMI 1640/5% BSA/10% FBS for 1 h. Peptides pools were diluted in culture medium (RPMI 1640 with L-glutamine, 10% cosmic calf serum (Hyclone, Logan, Utah), 10^5 M β-mercaptoethanol, 10 mM HEPES, 1× penicillin–streptomycin and 1× non-essential amino acids), and 50 μl of diluted peptide pools were added to the anti-mouse IFN-γ antibody-coated 96-well ELISpot plates to obtain a final concentration of 5 ng/ml (each peptide per pool). Then, an equal volume of splenocyte suspension from each animal at 4×10^6 cells/ml was added to corresponding wells at a final concentration of 2×10^5 cells/well, in duplicate. After an 18-hr incubation at 37 °C, the cells were lysed with water, and plates were washed extensively with PBS/T (PBS with 0.1% Tween-20) to remove cellular debris. A biotinylated anti-mouse IFN-γ secondary antibody (BD Biosciences) was added and allowed to incubate at room temperature for 1 h. Following an additional wash with PBS/T, alkaline phosphatase-labeled avidin (Sigma) was added for 1 h and the plates were washed again. Lastly, BCIP/NBT substrate solution (Pierce, Woburn, MA) was added and spots quantified using an AID ELISPOT reader (Autoimmun Diagnostika GmbH, Strassberg, Germany).

2.11. In vivo systemic toxicity assay

Mice were injected i.p. with 1×10^8 pfu of either cAdVaxM(ci), cAdVaxM(ra), cAdVaxM(mu), or HC4 on weeks 0, 4, and 8. Vaccinated mice were visually monitored daily for any adverse effects resulting from immunization. Animals were visually inspected for health, with particular attention to food and water intake, coat texture (ruffled coats are often a sign of illness), and excessive weight loss or gain. On week 12, internal organs were harvested from mice for histological examination. Organs from unvaccinated mice of the same age and average weight were harvested as a control. Tissues were fixed in 10% formaldehyde solution, paraffin-embedded and then cut into 5 μm sections. Paraffin sections were stained

with hematoxylin and eosin (H&E), following current histological methods, and examined for signs of toxicity.

3. Results

3.1. Construction of MARV vaccine vectors—adenovirus expression vectors that contain MARV sequences

We developed three cAdVax-based MARV vaccine vectors that express MARV GP genes for the purpose of developing effective and safe Marburg virus vaccines. These vectors were based on a replication-defective adenovirus vaccine vector platform that differs from other adenovirus-based vectors in that it contains multiple deletions within the adenoviral E1, E3, and E4 (except open reading frame 6, ORF6) genes and multiple insertion sites in the adenovirus genome [15–17]. These modifications enable the vector to accommodate relatively large amounts of exogenous DNA (up to 7 kb) and render the vector deficient for replication (see Fig. 1).

We have constructed a series of cAdVax-based MARV vaccine candidates known as cAdVaxM(ci), cAdVaxM(ra), and cAdVaxM(mu). Each vaccine construct expresses MARV GP from a single MARV strain. The cAdVaxM(ci) vaccine expresses Ci67 GP; cAdVaxM(ra) expresses Ravn GP; and cAdVaxM(mu) expresses Musoke GP. Each Ad-based MARV vaccine expresses two copies of their respective GP antigens, and each GP gene is under the control of the strong, constitutively active human cytomegalovirus intermediate/early (hCMVie) promoter. Several rounds of single-plaque isolation and purification were done to ensure that the vaccine preparations were homogeneous and free from

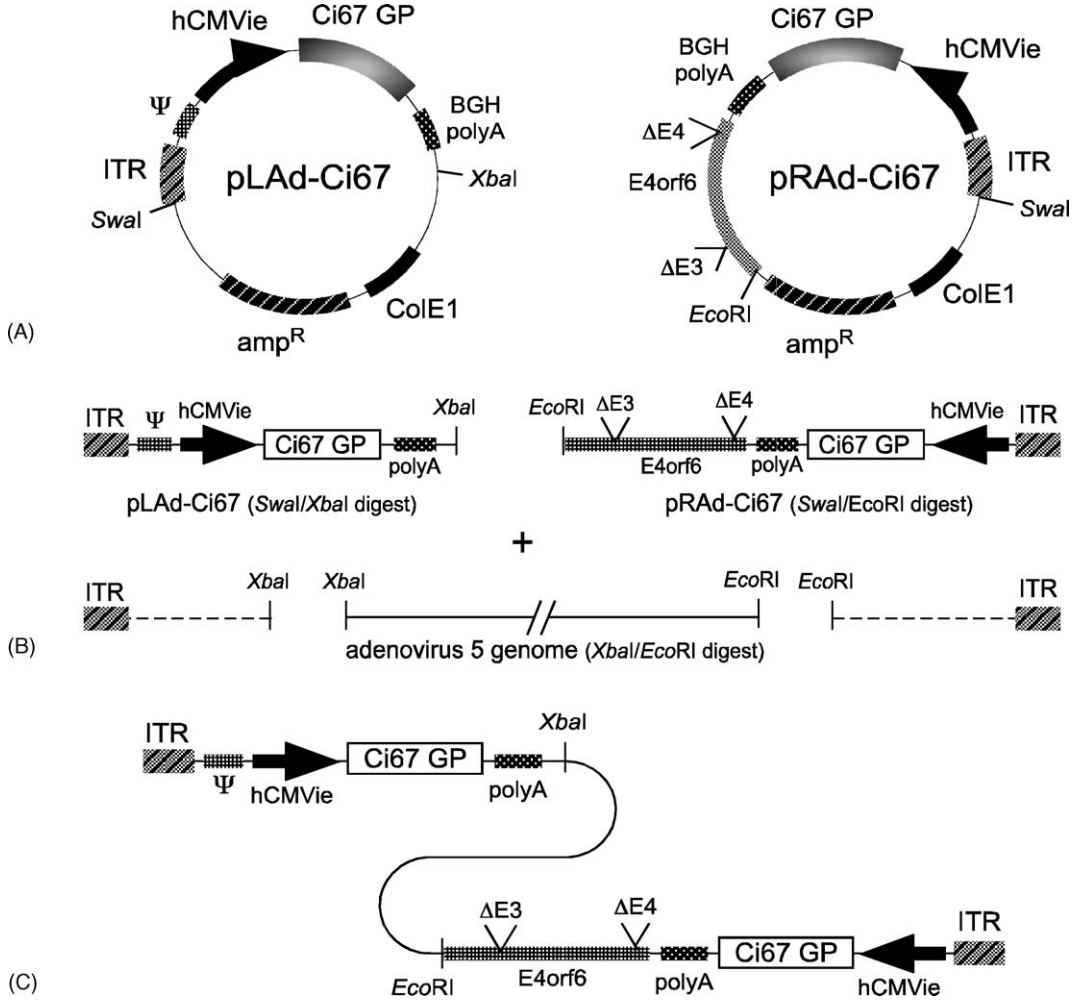

Fig. 1. Construction of the adenovirus-based vaccine vectors. Ad-based MARV vaccine vectors were constructed to express multiple MARV genes. (A) pLAd and pRAd shuttle plasmid vectors. A single strain of MARV GP (Ci67, Ravn, or Musoke) was cloned into both a pLAd (left adenovirus) and a pRAd (right adenovirus) plasmid vector, in which each GP was placed under the control of a human cytomegalovirus intermediate-early (hCMVie) promoter. (B) Ligation of restriction enzyme-digested vector fragments for the assembly of the adenovirus vaccine genome. pLAd and pRAd shuttle plasmids were digested with *Swa*I/*Xba*I and *Swa*I/*Eco*RI, respectively, while the adenovirus 5 genome was digested with *Eco*RI/*Xba*I. Gel-extracted restriction fragments were then ligated together to form the linear adenovirus vector genome. (C) Vector map of the final vector vaccine genome when completely constructed. Once the vector genomes were constructed, the ligation products were then transfected into HEK293 cells for vaccine vector selection and subsequent propagation and purification.

contaminants. Vectors were purified using density gradient centrifugation according to previously established protocols [15–17]. Purified preparations were titrated on HEK293 cells to determine their infectious activity and scored as pfu per ml. By design, each of these vaccines, upon transduction, should be able to induce cellular expression of their respective MARV antigens, without the consequence of expressing the vector components (i.e., adenovirus proteins).

3.2. High levels of MARV GP expression de novo mediated by adenovirus vectors

After completing construction of the vaccine vectors, it was important to verify that they induce MARV GP expression upon infection. To characterize the vaccines with respect to MARV GP expression, Vero cells were infected with the MARV vaccines at MOI 100. At day 2 post-infection, Triton X-100 detergent lysates of the infected cells were prepared. Cell lysates were resolved on 4–15% gradient polyacrylamide gels under denaturing and reducing conditions and the proteins were transferred to PVDF membranes. MARV GP proteins were detected by Western blot using a MARV-specific polyclonal guinea pig anti-Ravn antibody as detection antibody.

The MARV vaccines were found to mediate high levels of GP expression upon in vitro infection of Vero cells (Fig. 2). The GP1 and GP2 subunit products were detected by Western blot and were found to migrate at the expected molecular weights of 160 kDa and 38 kDa, respectively [6]. This suggests that the vaccine vectors successfully induced a de novo synthesis of their respective GP products, and that these proteins were properly processed and cleaved into the expected GP1 and GP2 subunits. The GP1/GP2 subunits displayed the typical appearance of a fully glycosylated protein, as the glycoprotein bands were found to be diffuse rather than sharp bands on the Western blot.

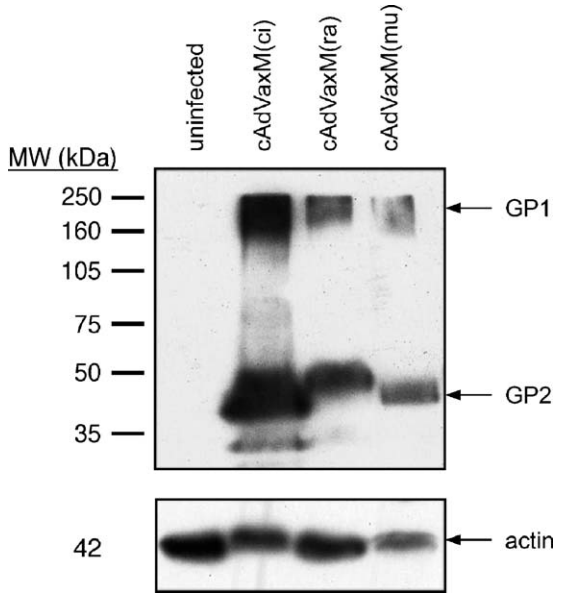

Fig. 2. Marburg vaccine candidates, cAdVaxM(ci), cAdVaxM(ra), and cAdVaxM(mu), demonstrate positive Marburg GP expression. Vero cells were infected with MARV vaccines, cAdVaxM(ci), cAdVaxM(ra), and cAdVaxM(mu). The cell lysates were resolved on 4–15% gradient polyacrylamide gel under denaturing and reducing condition. Membranes were probed with a polyclonal guinea pig MARV virus antibody. Lane 1: uninfected Vero lysate control; lane 2: cAdVaxM(ci) infected cells (Ci67 GP vaccine); lane 3: cAdVaxM(ra) infected cells (Ravn GP vaccine); lane 4: cAdVaxM(mu) infected cells (Musoke GP vaccine). Precursor and processed GP proteins are indicated to the right of the blot. GP1, 160 kDa; GP2, 38 kDa.

3.3. Vaccination with cAdVax-based MARV vaccine constructs produced humoral immune responses against MARV

In theory, cAdVax transduction of cells should induce a natural expression of the GP on host cell surfaces. This cell surface expression should mimic the presentation of antigens that would normally occur in a natural viral infection. Presentation of MARV antigen to the immune system should, therefore, induce both humoral and cell-mediated immune responses to MARV, without the damaging consequences of an actual MARV infection. In order to determine whether the de novo synthesis of viral GP by our cAdVax-based vaccines would mediate MARV-specific immune responses, we vaccinated C57BL/6 mice i.p. with 1×10^8 pfu per vaccine of cAdVaxM(ci); cAdVaxM(ra); or cAdVaxM(mu). As a negative control, one group of mice was immunized with 1×10^8 pfu of a cAdVax-based Hepatitis C vaccine, known as HC4, which induces Hepatitis C and not MARV antigens. Mice were boosted with 1×10^8 pfu of their respective vaccines at weeks 4 and 8 from the primary immunization. Sera and splenocytes from vaccinated mice were harvested biweekly for antibody and cytotoxic T lymphocyte (CTL) analyses. Based on dose response experiments, vaccination of mice with 1×10^8 pfu of cAdVax vaccine is sufficient to induce antibody titers to maximal levels (data not shown).

With the repeated dosing schedule we chose, we would expect an initial primary antibody response after the first injection followed by a considerable increase in antibody titer after either the first or second boost, indicating induction of a secondary immune response. In order to assay for induction of MARV-specific antibody, we conducted an anti-MARV ELISA, with inactivated MARV-Musoke virus serving as immune target. As shown in Fig. 3, each of the MARV vaccines induced strong antibody responses against inactivated MARV-Musoke. In addition, secondary immune responses were evident for all vaccines, particularly after the second boost at week 8.

Interestingly, cAdVaxM(ci) and cAdVaxM(ra) induced high levels of cross-reactive antibodies against MARV-Musoke virus even though cAdVaxM(ci) and cAdVaxM(ra) expressed MARV-Ci67 GP and Ravn GP, respectively. Additionally, the anti-Musoke antibody titers induced by the cAdVaxM(ci) and cAdVaxM(ra) vaccines were higher than those induced by the homologous cAdVaxM(mu) (Musoke) vaccine. Finally, anti-MARV antibodies were not detectable

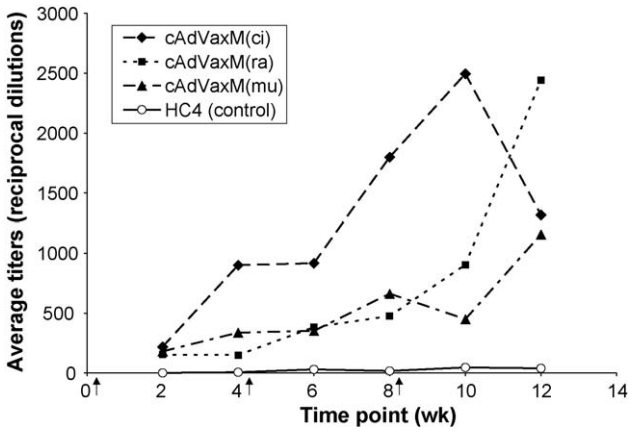

Fig. 3. Immunization of mice with cAdVax-based MARV vaccines induces antibodies specific for binding to inactivated MARV-Musoke virus. C57BL/6 mice were vaccinated i.p. with MARV vaccines, cAdVaxM(ci), cAdVaxM(ra), cAdVaxM(mu), and control (HC4), at 1×10^8 pfu per animal at weeks 0, 4 and 8 (Arrows indicate vaccination time points). At every 2-week interval, four animals were sacrificed. Each animal was analyzed individually for anti-MARV specific antibodies. Irradiated MARV-Musoke virus served as antigen for coating the ELISA plates. Titers were defined as the dilution of serum that produced a positive signal at 3× the background. Each point represents the mean of four individual samples.

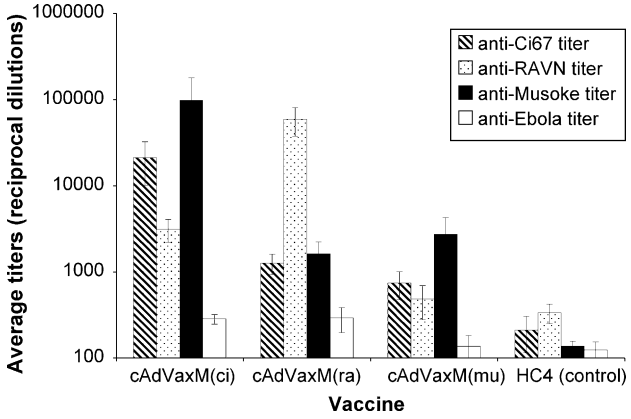

Fig. 4. Antibodies induced by MARV vaccine vectors demonstrate cross-reactive binding to heterologous MARV strains but not to the closely related filovirus, EBOV. Sera collected on week 24 during the experiment described in Fig. 3 were assayed for Ci67-, Ravn-, and Musoke-specific antibodies by ELISA. Additionally, sera were also assayed for Ebola virus-specific antibodies. Titers were defined as the dilution of serum that produced a positive signal at 3× the background. Each bar represents the mean of four individual samples.

in sera from HC4-vaccinated animals, indicating that the immune response induction by the cAdVaxM(ci), cAdVaxM(ra), and cAdVaxM(mu) vaccines were specific to MARV and not the adenoviral vector.

3.4. GP induced antibody responses are unevenly cross-reactive among three serotypes

Since the ELISA data described in the MARV-Musoke ELISA (Fig. 3) suggested that cAdVaxM(ci) and cAdVaxM(ra) vaccines might induce antibodies cross-reactive to heterologous strains of MARV, we conducted additional ELISA assays against each of the three strains for which the cAdVax-based vaccines were designed (i.e., Ci67, Ravn and Musoke). As a control, we additionally, conducted a fourth ELISA against a filovirus family member, Ebola virus, to determine whether the cAdVaxM(ci), cAdVaxM(ra) and cAdVaxM(mu)-induced antibodies were not only cross-reactive among MARV strains but might be cross-reactive to other filoviruses as well.

Results from these ELISAs supported the cross-reactive antibody response data seen in the original MARV Musoke-ELISA. As demonstrated in Fig. 4, the cAdVaxM(ra) and cAdVaxM(mu) vaccines both induced the highest levels of MARV-specific antibodies against their respective strains, but also induced significant cross-reactive antibodies to the other strains. These cross-reactive antibodies were shown to be specific to MARV as their activities were much higher than background antibody responses induced by the negative control HC4 vaccine. Interestingly, while the antibodies induced by the cAdVaxM(ci) vaccine were higher against Ci67 than to Ravn and Ebola, its highest antibody titers were specific to the Musoke strain. The antigenicity of the cAdVaxM(mu) vaccine appeared to be considerably weaker than the cAdVaxM(ci) and cAdVaxM(ra) vaccines. This seems to be consistent with previous studies conducted at USAMRIID, where Musoke appeared to demonstrate weak antigenicity in these studies as well. Finally, while antibody responses induced by the cAdVaxM(ci), cAdVaxM(ra), and cAdVaxM(mu) vaccines cross-reacted with other MARV strains, they were not cross-reactive against Ebola virus (Zaire 95 strain), even though Ebola virus is a close relative to MARV. These results suggest a broadly cross-reactive induction of antibody responses which are cross-reactive to other strains within the MARV species but which do not cross-reactive with other closely related members of the filovirus family.

3.5. Antigen synthesis de novo induced CTL responses to MARV GP antigen

Cytotoxic T lymphocytes destroy virus-infected cells and prevent production of nascent viruses by the infected host cells. For these reasons, induction of a productive cellular immune response against a viral pathogen is desirable for the development of a protective vaccine. In order to analyze the cellular immune responses induced by our MARV GP vaccines, we developed an IFN-γ ELISpot assay using overlapping peptide pools (15-mer peptides with a 10 amino acid overlap) derived from the Ci67 GP sequence as the CTL target.

As demonstrated in Fig. 5, all three vaccines demonstrated strong induction of CTL responses in inoculated mice. These cellular responses were specific to MARV, as the HC4-vaccinated mice failed to induce a CTL response to the Ci67 GP. In addition, a primary and secondary immune response induction was evident with all three vaccines in which a

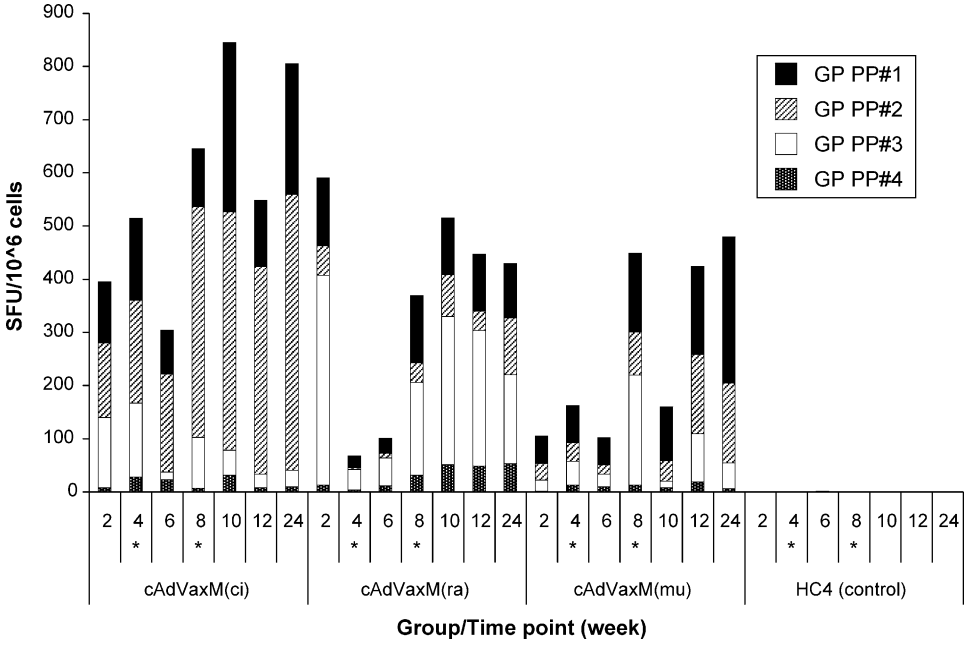

Fig. 5. cAdVax-based MARV vaccines induce MARV-specific CTL responses. Splenocytes collected during the experiment described in Fig. 3 were analyzed for CTL activity by IFN-γ ELISpot assay (* indicates booster vaccinations). Four peptide pools were generated using Ci67 GP sequence, and recognized the conserved regions of Ci67, Ravn and Musoke GP at their 5′ N- and 3′ C-termini. Peptide pools 1 and 2, GP PP1 and GP PP2, respectively, recognize the amino-terminus region of MARV GP1 protein at position a.a. 28–217, yielding two pools of 20 peptides each. Pools 3 and 4, GP PP3 and GP PP4, respectively, recognize the carboxy-terminus of GP2 at position a.a. 502–681, yielding two pools of 20 and 14 peptides, respectively. A pool of similarly constructed 15-mer peptides from the E protein of dengue virus type 2 was used as a negative control and concanavalin-A was used as a positive control. CTL activities are represented as spot-forming units per 1×10^6 cells.

noticeable increase in IFN-γ spot-forming units was observed from weeks 6 to 8 (Fig. 5). The cAdVaxM(ci)-vaccinated mice demonstrated the highest level of CTL stimulation by the peptide pools, particularly by the peptide GP PP1 and GP PP2 pools. This is to be expected since cAdVaxM(ci) includes two copies of Ci67 GP DNA, and the peptide pool targets were derived from Ci67 GP. Interestingly, cAdVaxM(ra) and cAdVaxM(mu) both induced strong CTL responses to the Ci67 peptide pools with cAdVaxM(ra) demonstrating particularly strong CTL activity against the GP PP3 peptide pool. These results suggest that the de novo synthesis of individual strains of MARV GPs by the cAdVaxM(ci), cAdVaxM(ra), and cAdVaxM(mu) vaccines may provide a broadly reactive CTL response against multiple strains of MARV.

3.6. Vaccination with multiple doses did not cause toxicity in mice

It has been shown in gene therapy studies that Ad vectors have a high affinity for the liver, and therefore, it is possible that vaccination with Ad-based vectors may cause inflammation in the liver. In order to assess the safety of our cAdVax-based vaccines, one group of mice injected with the same dosing schedule as the mice from the antibody and CTL studies were sacrificed on week 12. Their organs were harvested and examined histologically for signs of toxicity. As a control, unvaccinated mice of the same age and average weight were also sacrificed. Their organs were also harvested for histological analyses.

We conducted extensive histological studies of vital organs of all vaccinated groups. In all animals examined, no detectable differences could be found between experimental groups and controls. As shown in Fig. 6, H&E-stained sections of both liver and spleen were similar in appearance and lacked pathology indicative of inflammation or cytotoxicity as a result of vaccination. In addition, all vaccinated and control mice were visually monitored daily for any adverse effects resulting from immunization. Particular attention was paid to general conditions, behavior, food and water intake, coat texture (ruffled coats are often a sign of illness), and excessive weight loss or gain. All mice appeared healthy.

4. Discussion

In this study, we have constructed and characterized three MARV vaccine candidates based on a novel replication-defective complex adenoviral vector, or cAdVax, system. Upon vaccination, these vaccine vectors can mediate high levels of antigen syntheses within the cells transduced at the site of vaccination. We hypothesized that by inducing MARV GP syntheses de novo, these antigens would retain the natural conformations and post-translational modifications of the native GPs, and therefore, would include intact viral

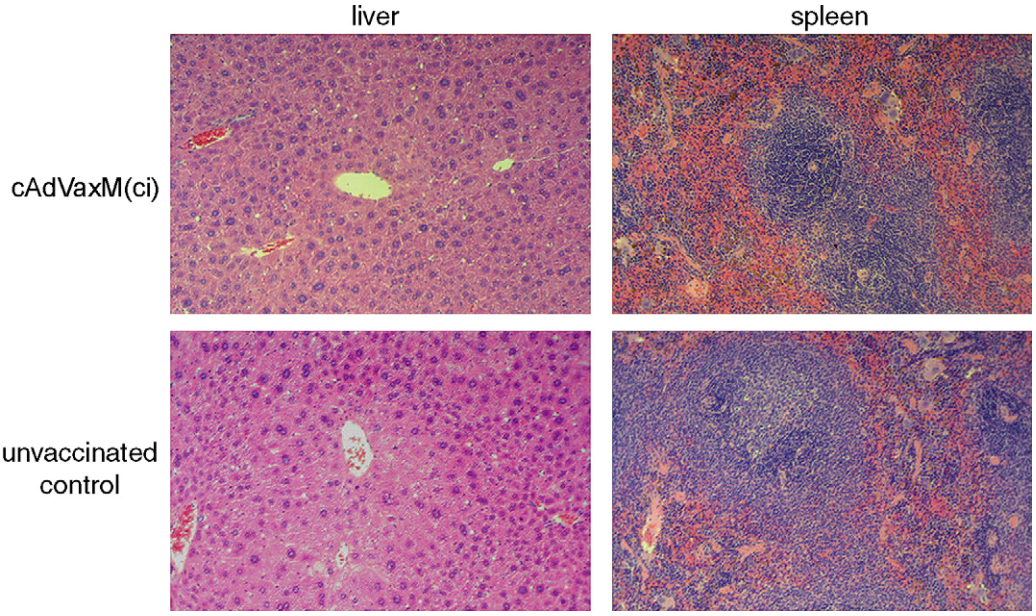

Fig. 6. Histological analyses of vaccinated mouse livers and spleens indicate a lack of toxicity following a three-dose vaccination schedule. C57BL/6 mice were injected i.p. with 1×10^8 pfu of either cAdVaxM(ci), cAdVaxM(ra), cAdVaxM(mu), or HC4 on weeks 0, 4, and 8, as described in Fig. 3. Mice were sacrificed on week 12 and their vital organs harvested for histological examination. Vital organs were also harvested from uninfected mice as a control. Tissues were fixed in 10% formaldehyde solution, paraffin-embedded and then cut into 5 μm sections. Paraffin sections were H&E stained following current histological methods and examined for signs of toxicity. The cAdVaxM(ci) sections shown were found to be representative of the lack of toxicity seen in the livers and spleens of the cAdVaxM(ra)- and cAdVaxM(mu)-vaccinated mice.

receptor-binding sites, in particular, the sites in which the viral neutralizing epitopes would be located. As a result, antibodies specific to the receptor-binding site would be produced which should neutralize subsequent viral infection by preventing viral entry into the host cells. In addition, the vaccine-expressed viral antigens can persist from days to weeks to provide long-lasting stimulation of the host immune system, and therefore, induce potent antibody and CTL responses.

In addition to humoral immune responses, we believe that induction of CTL responses would be important in protection against deadly pathogens as well. CTLs play important roles in destroying infected host cells and in preventing the replication and release of nascent viruses. We believe the naturally expressed antigens on cell surfaces will mimic a natural infection by the pathogenic virus without actually causing the disease. This is a major advantage of the cAdVax-based vaccines over protein-based subunit vaccines that are not glycosylated and not correctly folded, or over recombinant protein antigens synthesized in eukaryotic cells that may initially possess the correct conformations but which may become degraded or destroyed in the extensive purification processes.

We have chosen the cAdVax system to transfer the genes encoding MARV antigens for many of its advantages: (1) This cAdVax vector contains several large deletions that allow for the incorporation of large amounts of exogenous DNA. (2) It is capable of expressing high levels of transgenes (e.g., antigens from viral pathogens) using expression cassettes without expressing adenoviral genes. This will direct immune responses mainly towards the targeted viral antigens and not the vector proteins. (3) In vivo syntheses of viral antigens provide long lasting stimulation (up to 2 weeks) of the immune system, and therefore, may need fewer vaccinations. (4) Ad-based vectors infect dendritic cells, a potent antigen presenting cell type. (5) cAdVax is replication-deficient, and therefore, enhances the safety of the vaccines. In gene therapy studies, other Ad5 vectors have been administered in high doses and have been instilled into vital organs, such as heart and lungs in >2000 patients [18–22]. Even replication-competent adenoviruses have been used safely as vaccines given to military recruits for the common cold, or "boot camp flu" [23,24]. (6) Ad vaccines are easy to produce and a number of commercial manufacturing facilities have been established. (7) The major difference between cAdVax and the early generation adenoviral vectors is that it has the capability of expressing multiple antigens from a single construct to induce immune responses against multiple antigens or multiple pathogens. Although this advantage has not been fully utilized in this first group of vaccine candidates described here, it will be applied to our future trivalent MARV vaccines after we have analyzed the importance of each strain antigen(s) in inducing type-specific immune responses.

To test our hypothesis that de novo synthesis of viral antigens via a cAdVax vector will be able to mimic a natural infection and induce potentially protective immune responses, we constructed the cAdVaxM(ci), cAdVaxM(mu), and cAdVaxM(ra) vaccine vectors, which express GP antigens of Ci67, Musoke and Ravn strains, respectively. Each vaccine,

upon transduction of Vero cells, was able to induce synthesis of its respective MARV GP in vitro. Based on Western blot analyses, these proteins appeared to be fully glycosylated (indicated by the diffuse rather than sharp protein band) and enzymatically processed into their mature GP1/GP2 subunits. Because these glycoproteins are naturally synthesized in cells using the same cellular secretory pathway as in cells infected by MARV, it is reasonable to assume that these MARV GPs are expressed in their natural conformation and retain their proper receptor binding sites, where the important neutralizing epitopes would be located.

Using murine vaccination models, we demonstrated that cAdVax-mediated expression of MARV GPs induced high levels of type-specific antibody responses. Furthermore, these antibody responses could be boosted with additional vaccinations. At least in our experimental conditions, the immunity against the adenoviral vector did not prevent subsequent vaccinations. In addition, after the boost vaccination, the antibody responses demonstrated a typical secondary immune response that increased rapidly within 2 days versus 2 weeks in the primary immune responses. This suggests that memory immune responses may have been activated, which are critical for long-term immunity.

Humoral immune responses are essential in protective immunity against MARV infection, as shown in various challenge models [12,14,25] and serologic studies of a few recovered patients [2]. However, neutralizing activities against MARV have been difficult to detect using the conventional plaque reduction assays that have been proven effective for other infectious viruses, such as flaviviruses. It is generally believed that neutralizing assays are not predictive of protection. However, a number of studies did show a close correlation between antibody levels in sera and protection against infections by MARV [14,25,26] or a closely related filovirus, EBOV [26–28]. Further evaluation will be necessary to determine whether the high antibody titers induced by our vaccines will provide protection against lethal MARV challenges.

Because each of our vaccines expressed only a single strain of MARV GP, we were able to study the immune responses against a specific strain and cross immunities against different strains. Interestingly, we have also shown significant levels of cross immune responses among different strains. However, the humoral immune responses are clearly MARV-specific as these antibodies did not react with EBOV. The pattern of cross immunity showed "uneven cross-reactivity". For example, vaccination with cAdVaxM(ci) showed high cross immunity against Musoke strain, but vaccination with cAdVaxM(mu) did not generate high cross immunity against either the Ci67 or Ravn strains. This suggested that additional factors, in addition to sequence homology between the strains, might play a role in the levels of cross immune responses. Although the levels of cross immunities were generally lower than direct immunities (with the exception that cAdVaxM(ci) vaccinations induced higher antibody responses against Musoke than its own strain (Ci67)), we have also found that Musoke GP consistently induced the lowest levels of antibody responses, both Musoke-specific and cross-reactive (Ci67- or Ravn-specific). While such differences in antigenicity are not unique to MARV or filoviruses, these data do appear to suggest that while the Musoke strain has previously served as the model strain for development of MARV vaccines [10–12,26], the Ci67 and Ravn strains may potentially be more effective at inducing cross-protective MARV-specific immune responses.

The significance of the cross immune responses in broad protection induced by vaccination is complicated to predict. It is not known, in a natural infection, whether patients who have recovered from one strain of MARV infection would develop protective immune responses against other strains, due to the high mortality of the infection and the rareness of these diseases. Studies of cross protection have been further complicated by the lack of an effective neutralizing assay. However, in animal models, it has been shown that cross protection has been difficult to achieve, as animals that have survived challenge of one strain would die when they were back-challenged (re-challenged with a different strain) [11]. We believe that the cAdVaxM(ci), cAdVaxM(mu), and cAdVaxM(ra) vaccines provides us with a powerful tool for examining the roles of each strain GP in inducing direct- and cross-humoral immune responses. Future co-vaccination and virus challenge studies, in which individuals are immunized with a mixture of cAdVaxM(ci), cAdVaxM(ra), and/or cAdVaxM(mu), may reveal the optimal combination of antigens needed for the induction of protective immunity against multiple strains of MARV. The optimal combinations of GP antigens may then be inserted into a single cAdVax vector to establish a multivalent MARV vaccine.

In addition to the humoral immune responses, we have also analyzed MARV-specific CTL responses. In general, the CTL arm of the immune system is also critically important in defense against virus infections. Activated T-lymphocytes play a major role in destroying infected cells, preventing viral replication, reducing viral load and eventually eliminating the infection. In the case of filovirus infections, mortalities often occur before sufficient time is allowed for the activation of CTL responses. However, in a few cases of survival, low levels of filovirus-specific CTL activities were detected, while no neutralizing activities were detected. We hypothesize that activation of a MARV-specific CTL response before exposure to MARV would give the activated CTLs a chance to establish themselves and proliferate quickly in the event of an infection. The resultant MARV-specific CTLs would be able to perform active roles in both destroying infected cells and preventing MARV from replicating in the infected cells. In addition, one of the benefits of CTL responses is that CTL epitopes tend to be highly conserved peptide regions of the protein antigen. As a result, CTL reactivities tend to be much broader than antibody neutralizing activities. Therefore, CTL responses may provide broad protection against multiple strains of MARV. In our study, we have shown that antigen synthesis de novo can effectively induce CTL

responses against MARV, based on ELISpot analyses. We attribute this to the persistent stimulation of the immune system by endogenously induced MARV antigen production and presentation. In combination with the humoral responses, we believe that these MARV-specific CTLs will play important roles in protective immunity against MARV.

Finally, we analyzed the safety profile of the vaccines in a murine model. After high dose (10^8 pfu) i.p. vaccination, we detected no abnormality in behavior or body signs in the vaccinated groups by visually inspecting the animals. Histological analyses of the vital organs, including liver and spleen, showed no differences between the vaccinated groups and the control groups. Liver is especially sensitive to adenovirus vector infection (transduction) and vector-induced inflammatory reactions when the vector is injected directly into blood. This is due to the anatomical structures of the hepatic portal vein system that allow hepatocytes direct contact with adenoviral particles circulating in the blood, in addition to the high density of adenovirus receptor (CAR) and coreceptors ($\alpha v\beta 3$ integrins) present on these cell types. It is possible that small numbers of cAdVax vectors could leak into the blood circulation and transduce the hepatocytes, but the number of transduced cells are small and do not induce a significant amount of inflammatory responses detectable by histological methods. In addition, liver has a high regenerative potential and high tolerance to damage. Taking together, while more extensive toxicological analyses will be necessary prior to conducting clinical trials with these vaccines, these data and our previous experience with cAdVax-based vaccines given at up to 10^9 pfu/mouse (data not shown) do appear to suggest that cAdVax-based MARV vaccines can be safely administered to mice at high dosages.

In summary, we have shown that de novo synthesis of MARV GP antigens with cAdVax vectors induced potent humoral and CTL responses against each of the three MARV strains for which they were designed. We have analyzed the antigenicities of each strain MARV GP and their abilities to induce cross immunity as well as demonstrated the safety of cAdVax-based MARV vaccines. Our data show that cAdVax-based MARV vaccines provide several advantages not offered by other candidate vaccines, such as the safety of a non-replicating subunit vaccine, efficient antigen delivery in the absence of additional vector gene expression, presentation of MARV GP antigens to the immune system in their native forms, and induction of a cross-reactive immune response to heterologous MARV strains. Our data have shown that the cAdVaxM(ci), cAdVaxM(mu), and cAdVaxM(ra) vaccines have excellent potential as candidate vaccines against MARV and may provide tools to further analyze protective immune responses against multiple strain MARV infections. Based on current and previous studies, it is likely that an effective trivalent vaccine would require the expressions of multiple strain GP antigens. These may be achieved by mixing two or three cAdVax vectors that express each of the GP proteins. Once we have a better understanding of the antigenic requirement for protective immunity, we will take full advantage of the multiple antigen capability of cAdVax to establish a single vaccine candidate that expresses the GP antigens of all necessary strains. This would be advantageous in simplifying the production and approval process of the vaccines.

Acknowledgments

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